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(54) Title: METHODS AND COMPOSITIONS FOR IDENTIFICATION OF AUTOANTIGENS

(57) Abstract

Autoantigens with immunocryptic sites may be cleaved at particular sites in the presence of metals such as iron or copper and reactive oxygen species to produce antigenic protein fragments which are useful in diagnosing autoimmune diseases. Substances that interfere with fragmentation process may be used to treat autoimmune diseases and the fragments may be used to tolerize patients. Non–enzymatic proteolysis according to the invention has wide applicability as a biochemical tool.

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METHODS AND COMPOSITIONS FOR IDENTIFICATION OF AUTOANTIGENS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of co-pending U.S. provisional application serial number 60/034,098 filed on December 30, 1996, and which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention generally relates to hydrolysis of certain proteins, and, more particularly, to methods and compositions for specifically cleaving autoantigens by metal-catalyzed proteolysis to form specified protein fragments. The present invention particularly relates to methods for detecting and preparing the protein fragments, methods for making antibodies against the protein fragments, assays employing such protein fragments and antibodies, and methods for treating autoimmune diseases modulated by autoantigens.

2. Background

The highly specific humoral immune response to autoantigens in many autoimmune diseases is antigen-driven and T cell-dependent (M. Radic et al., *Ann. Rev. Immunol.*, **12**:487-520 (1994); B. Diamond et al., *Ann. Rev. Immunol.*, **10**:731-757 (1992)), but the initial mechanisms for breaking cell tolerance to these molecules remain unclear. Several recent studies demonstrate that a potential for T cell autoreactivity resides in the immunological non-equivalency of different areas of self-molecules, since self-tolerance is only induced to efficiently presented, dominant epitopes, but not to cryptic ones (reviewed in E. Sercarz et al., *Ann. Rev. Immunol.*, **11**:729-766 (1993); A. Lanzavecchia, *J. Exp. Med.*, **181**:1945-1948 (1995)). Thus, potentially autoreactive T cells that have not previously encountered the cryptic self still exist (G. Gammon et al., *Nature*, **342**:183-185 (1989)). As determinant dominance is influenced by protein structure, circumstances that change the molecular context of epitopes (e.g. novel cleavage, altered conformation or tertiary structure) may permit the efficient presentation of previously cryptic determinants, thereby

breaking T cell tolerance (M. Mamula, *J. Exp. Med.*, **177**:567-571 (1993); L. Bockenstedt et al., *J. Immunol.*, **154**:3516-3524 (1995); S. Salemi et al., *J. Exp. Med.*, **181**:2253-2257 (1995); C. Watts et al., *J. Exp. Med.*, **178**:1459-1463 (1993); P. Simitsek et al., *J. Exp. Med.*, **181**:1957-1963 (1995)). The unique autoantibody response observed in different autoimmune diseases may therefore be viewed as the long-lived immunologic memory of the altered circumstances that revealed this cryptic structure. These antibodies are thus useful probes with which to search for the initial perturbed state.

For example, the autoantibodies elaborated in systemic lupus erythematosus (SLE) have fused attention on apoptosis as a possible setting in which cryptic structure is revealed. During apoptosis, the lupus autoantigens cluster and become concentrated in the surface blebs of apoptotic cells (L. Casciola-Rosen et al., *J. Exp. Med.*, **179**:1317-1330 (1994)), where several of these molecules are specifically cleaved by proteases of the interleukin 1 (converting enzyme (CE) family (L. Casciola-Rosen et al., *J. Biol. Chem.*, **269**:30757-30760 (1994); L. Casciola-Rosen et al., *J. Exp. Med.*, **182**:1625-1634 (1995); L. Casciola-Rosen et al., *J. Exp. Med.*, **183**:1957-1964 (1996)). The fact that specific proteolytic cleavage unifies these lupus autoantigens has suggested that fragmentation might define molecules as autoantigens in other autoimmune diseases (L. Casciola-Rosen et al., *J. Exp. Med.*, **182**:1625-1634 (1995)).

Scleroderma is a disease of unknown etiology which is characterized by increased vasoreactivity, widespread tissue fibrosis and the elaboration of unique autoantibodies. Since the autoantigens recognized are not substrates for the ICE-like enzymes during apoptosis, it is likely that other mechanisms are responsible for revealing cryptic structure in this disease (L. Casciola-Rosen et al., *J. Exp. Med.*, **182**:1625-1634 (1995)). One potential mechanism that might result in the specific fragmentation of scleroderma autoantigens is suggested by the striking reversible ischemia-reperfusion that occurs in patients with scleroderma (reviewed in (J. Belch, *Annals. Rheum. Dis.*, **50**:839-845 (1991)). This vascular phenomenon, the result of dysfunction of small arteries and arterioles of the extremities and internal organs, has been proposed to underlie the exuberant tissue fibrosis in this disease (W. Norton et al., *Ann. Intern. Med.*, **73**:317-324 (1970)). The injury associated with reperfusion of

ischemic tissues results in part from the production of free radical species (J. McCord et al., *N. Engl. J. Med.*, **312**:159-163 (1985); J. Zweir, *J. Biol. Chem.*, **263**:1353-1357 (1988); C. Oliver et al., *Proc. natl. Acad. Sci. USA*, 87:5144-5147 (1990)), and indirect evidence exists for increased production of reactive oxygen species (ROS) in patients with scleroderma (A. Herrick et al., *J. Rheumatol.*, **21**:1477-1483 (1994); C. Stein et al., *Arthritis Rheum.*, **37**:1146-1150 (1996)). It is known that ROS can induce the oxidative modification of proteins (including fragmentation (K. Davies, *J. Biol. Chem.*, **262**:9895-9901 (1987); E. Stadtman, *Free Radic. Biol. Med.*, 9:315-325 (1990)). It is also known that D-penicillamine appears to improve the outcome patients with diffuse scleroderma, when used for extended periods (V. Steen et al., *Ann. Intern. Med.*, 97:652-659 (1982); S. Jimenez et al., *J. Rheumatol.*, **18**:1496-1503 (1991)).

Neely, U.S. 5,504,090 describes a method for preventing ischemia-reperfusion injury in organ by use of a selective A1 adenosine receptor antagonist. Wu, U.S. 5,248,668 and, 5,047,395, discloses the use of purpurogallin and bilirubin derivatives as antioxidant agents to minimize oxyradical damage caused by ischemia-reperfusion injury. Stanko, U.S. 5,480,909 relates to the use of pyruvate to inhibit free radical generation and to scavenge free radicals. Lipid peroxidation and radical formation were reduced by inositol triphosphate according to Siren, U.S. 5,019,566.

Meruelo et al., U.S. 5,514,714, describes the use of hypericin to treat T cell-mediated autoimmune diseases. Furanose monosaccharides were said to be effective against auto immune and proliferative diseases in Ronsen et al., 4,996,195, and dipyridamole was described as having similar use in Kauffman et al., U.S. 5,314,688. Inhibitors of angiotensin converting enzyme is said to be useful for treating vascular conditions including scleroderma, according to Loscalzo et al., U.S. 5,025,001. A diphenyl sulfone is taught as having therapeutic effectiveness against scleroderma in Goloschapov et al., U.S. 4,151,281. Aris et al., U.S. 5,310,892, describes the diagnostic use of fibrillarin to detect anti-fibrillarin antibodies in scleroderma patients.

There are currently several ways to assay for scleroderma autoantigens, including Ouchterlony immunodiffusion, ELISA, and western blotting. The assay of the autoantigens targeted in scleroderma is an insensitive and laborious process, and several autoantigens that are potentially useful as diagnostic and prognostic markers

have therefore not had utility in routine clinical practice. For example, use of immunoblotting has not been possible, due to problems with standardization and determining specificity. There is a need for technologies with greater sensitivity and specificity.

SUMMARY OF THE INVENTION

The present invention generally relates to methods and compositions for detecting autoantigens. In one aspect, the present invention relates to therapies for treating or preventing various autoimmune diseases modulated by undesired fragmentation of normally tolerized autoantigenic proteins. In particular, therapies have been identified that include preventing or inhibiting production of the protein fragments.

More specifically, the invention features methods for treatment of autoimmune diseases, particularly those diseases modulated by formation of autoantigenic and immunogenic protein fragments from the tolerized autoantigens. Examples of such diseases include but are not limited to scleroderma, lupus, Sjogren's syndrome, polymyositis, dermatomyositis, multiple sclerosis, type I diabetes mellitus, rheumatoid arthritis, and mixed connective tissue disease (MCTD). The methods involve reducing or eliminating formation of the fragments by inhibiting metal-catalyzed oxidative proteolysis of autoantigenic proteins, e.g., certain nucleic acid related proteins, that have been implicated in the development or severity of various autoimmune diseases.

As will be discussed in more detail below, we have discovered that certain autoantigens are capable of focussing metal-catalyzed oxidative proteolysis reactions to sites on the autoantigens particularly metal binding sites. In general, the reactions catalyze formation of reactive oxygen species (sometimes referred to herein as ROS). It has been found in accord with the present invention that the ROS can specifically cleave the autoantigens to produce the autoantigenic and immunogenic protein fragments.

Therapeutic methods of the invention can be employed to prevent or inhibit the metal-catalyzed proteolysis reactions that facilitate production of the fragments. The methods generally include administering to a subject, particularly a mammal such as a primate, especially a human, a therapeutically effective amount of a compound

(sometimes referred to herein as a therapeutic compound or substance) that can reduce or eliminate metal-catalyzed oxidative proteolysis of a specified autoantigen. Preferably, an administered compound inhibits the proteolysis by at least about 10% or 25% as determined, e.g., by a standard protein fragmentation detection assay. Specific examples of such assays are described below. It is generally preferred that the administered compound be capable of inhibiting production of the protein fragments by at least 30%, 40%, 50%, 70%, 80% or 90% or more as determined by a standard immunological assay. Illustrative examples of such assays include Ouchterlony immunodiffusion, ELISA, or other suitable immunoassay capable of detecting production of immunogenic protein fragments. A particularly preferred assay is a Western immunoblot. Preferably, a suitable protein fragmentation detection assay can be employed to quantitate protein fragments. Additionally preferred assays include one or more suitable controls such as those specified below.

Compounds suitable for use in accord with the treatment methods of the invention include compounds that are capable of inhibiting or eliminating metal-catalyzed oxidative proteolysis. Preferred compounds inhibit metal-catalyzed production of ROS such as those reactive oxygen species produced by Fenton or Haber-Weiss related chemical reactions such as those discussed below. Examples of such inhibitory compounds include metal chelators, as well as certain metals such as zinc that have been reported to inhibit the Fenton or Haber-Weiss reactions.

Preferred compounds exhibit an IC_{50} of at least about 0.1μM to 200μM in a standard metal-catalyzed Fenton or Haber-Weiss reaction as defined below, more preferably an IC_{50} of about 1μM to 50μM, still more preferably about 5μM to 20μM, and even more preferably an IC_{50} of about 15μM in the standard reaction. Suitable compounds in accord with the invention are sometimes referenced as "proteolytic inhibitor compounds" or like term or phrase to denote capacity to inhibit the metal-catalyzed oxidative proteolysis reactions.

Specifically preferred compounds are capable of binding an iron- or copper-catalyzed Fenton or Haber-Weiss reaction, e.g., desterroxamine, D-penicillamine, ethylenediamine tetraacetic acid (EDTA), bathocuproine disulfonate and related compounds.

Other suitable compounds can be readily identified by simple testing in accord with the invention, e.g., by *in vitro* testing of a candidate inhibitor compound relative to a suitable control for capacity to inhibit formation of autoantigenic and immunogenic protein fragments, e.g., by at least 10% or more relative to the control.

Although usually well-suited for inhibiting autoantigen fragmentation in an *in vitro* assay of the invention, additional compounds capable of reducing or eliminating coordination a metal and one or more accessible amino acid groups on the autoantigen are contemplated. Preferred compounds are those capable or reacting with accessible carboxylate groups, e.g., 1-ethyl-3-(3-dimethlyaminopropyl) carbodiimide (EDAC).

Compounds of the invention can be used to diagnose and monitor autoimmune conditions, and to assay the effectiveness of treatments for such conditions. Moreover, by preventing or inhibiting the production of such fragments, the severity or extent of injury from autoimmune diseases may be reduced. For example, the autoantigenic protein fragments may be used as tolerizing reagents in the prevention and therapy of autoimmune diseases as set forth in the detailed description.

Particular treatment methods may be used to treat or reduce severity of scleroderma. In this instance, the autoantigen may be topoisomerase I, the large subunit of RNA polymerase II, upstream binding factor (UBF/NOR90), or the 70kDa protein component of the U1 small nuclear ribonucleoprotein, or any antigenic peptide, and the substance may be a chelator, metal other agents that compete with the iron or copper, or other inhibitors of Fenton oxidation and metal-mediated oxidation of proteins.

Preferably a chelator according to the invention is less toxic and more effective than known substances such as D-penicillamine. The proposed mode of action of D-penicillamine the prior art is inhibition of the Cu-requiring enzymatic reaction required for the cross-linking of collagen. The studies here demonstrate that the unique fragmentation of scleroderma, autoantigens by metal-catalyzed oxidation reactions is a critical target in scleroderma, and prompts an entirely different strategy for identification of relevant agents (i.e. agents that have high nucleolar concentrations and effectively inhibit the Fenton reaction). Preferred chelators include desferroxamine and bathocuproine disulfonate.

Additional particular treatment methods according to the invention include administering a therapeutically effective amount of one or more autoantigenic and immunogenic protein fragments of the invention over a period of time sufficient to substantially or completely tolerize the patient to an autoimmune disease. Further, the methods can be used prophylactically to prevent onset of an autoimmune disease in a subject susceptible or believed to be susceptible to such a disease.

Preferably, the autoantigenic and immunogenic protein fragments are produced in accord with the present invention, ie. by facilitating metal-catalyzed oxidative proteolysis *in vivo* or *in vitro*.

The invention further relates to methods of producing autoantigenic and immunogenic protein fragments of normally tolerized autoantigens by metal-catalyzed oxidative proteolysis reactions as generally described above. Preferably, the fragments are produced in the presence of metals which are sometimes referred to herein as "Fenton metals" to denote capacity to facilitate oxidative proteolysis in accord with Fenton or Haber-Weiss type chemical reactions discussed below. As noted above, these chemical reactions produce ROS and focus same to specific autoantigen sites resulting in autoantigen cleavage. Particularly preferred examples of Fenton metals include iron and copper. A Fenton metal is preferably used as a suitable salt as specified below.

In particular, certain autoantigens from scleroderma patients and patients with other autoimmune diseases have in common a susceptibility to metal-dependant ROS-mediated cleavage of the proteins into fragments. These antigenic protein fragments are believed to be related to those which are the targets of the immune system in these patients. As will be discussed below, the invention also facilitates easy identification and quantitation of autoantibodies recognizing these autoantigens (such as with scleroderma: topoisomerase I, RNA polymerase II, U1-70kDa and NOR90). Since only sera from the diseased patients recognize the novel fragments generated by this chemical proteolysis, and the levels of autoantibodies reflect the severity of the autoimmune process, the invention is useful for early and specific diagnosis of auto immune patients, such as scleroderma patients, as well as the serial evaluation of disease severity to guide therapy.

Diagnosis of other autoimmune diseases is readily accomplished according to the invention, by cleaving autoantigens particular to those diseases via metal-mediated oxidation, and determining whether a patient's serum cross-reacts with the autoantigenic protein fragments.

The field of diagnosis and treatment of autoimmune diseases is crowded and has not provided much success. Antioxidants have proved to be of limited therapeutic use. There is a pressing need for new technologies. This invention succeeds where previous efforts of specifically and repeatably generating antigenic epitopes from autoantigens have failed. By cleaving autoantigens according to the invention, cryptic antigenic sites are revealed. The use of chemical proteolysis to achieve this result was not previously known or suggested. The advantages presented by the fragments produced according to the invention could not have been appreciated before this invention. The method of metal-mediated protein cleavage also has wide-ranging applications in protein biochemistry, and could be readily adapted for use on an industrial scale as an alternative to enzymatic proteolysis.

The autoantigenic and immunogenic protein fragments of the present invention usually include one or more cryptic epitopes revealed by the metal-catalyzed oxidative proteolysis and which are typically immunologically detectable. Molecular weights the fragments are influenced by several parameters including desired efficiency or completeness of a metal-catalyzed oxidative proteolysis reaction. As will be discussed below, the molecular weight is particularly effected by the number and location of metal binding sites in the autoantigen. Generally, most protein fragments will have a molecular weight in the range of between about 10kDa to 500kDa, more typically 30kDa to 250kDa as determined by standard molecular sizing techniques such as chromatography and electrophoresis. See the discussion and examples below.

The invention provides advantages that can be exploited in the understanding, diagnosis and therapy of autoimmune diseases. Such advantages include use of autoantibodies, e.g., as probes for analyzing initial stimulation of the immune system in different autoimmune diseases. Additionally, protein fragmentation is an efficient method to initiate an immune response to self-molecules. In particular, autoantigenic protein fragments produced in accord with the invention can be used to raise

antibodies, particularly commercially. As illustrated in the examples which follow, the fragments can also be used in diagnostic assays to detect certain autoimmune diseases, e.g., scleroderma, MCTD, lupus, Sjogren's syndrome, polymyositis, dermatomyositis, multiple sclerosis, type I diabetes mellitus, rheumatoid arthritis and others.

This invention applies to nearly any autoimmune disease where autoantigenic fragments are revealed by a metal catalyzed reaction. Scleroderma (diffuse and limited, including the CREST variant), and MCTD are two examples of such diseases. In addition, experimental evidence shows that similar principles apply in the case of rheumatoid arthritis, and in fibrosing alveolitis spectrum of lung disease.

The invention also relates to a composition useful in reducing injury from scleroderma comprising an effective amount of a substance that inhibits the fragmentation of autoantigens into peptides in the presence of iron or copper and reactive oxygen species. Administration may be intravenous or otherwise as indicated below.

As noted, the antigenic protein fragments of the invention have defined molecular weight. Additionally, such fragments cross-react with serum from a patient for the autoimmune disease in question, and have varying degrees of immunogenicity in the sense of eliciting an immunogenic response in an individual without the autoimmune disease. For example, with scleroderma, RNA polymerase has greater specificity of binding and is more antigenic than some of the other antigenic protein fragments produced according to the invention.

According to another aspect of the invention, autoantigenic and immunogenic protein fragments of normally tolerized antigens may be produced in the presence of specified metals and ROS.

Illustrative methods of making autoantigenic and immunogenic protein fragments according the invention are as follows. For example, a method scleroderma autoantigens according to the invention comprises obtaining a protein selected from the group consisting of topoisomerase I, the large subunit of RNA polymerase II, upstream binding factor (UBF/NOR90), and the 70kDa protein component of the U1 small nuclear ribonucleoprotein, and fragmenting the protein in the presence of iron or copper and reactive oxygen species. Scleroderma autoantigen fragments according

to the invention may be topoisomerase I fragments migrating at 95kDa, and species migrating between 65kDa and 90kDa; fragments of the large subunit of RNA polymerase II migrating at 190, 160 and 140kDa, and fragments migrating at 200, 180, 170 and 130kDa; fragments of the upstream binding factor (UBF/NOR90) migrating 70kDa and 90kDa; and fragments of 70kDa protein component of the U1 small nuclear ribonucleoprotein (U1-70kDa) migrating between 33 and 3kDa.

Yet another method of making antigenic protein fragments in accord with the invention comprises reacting autoantigens for the disease in the presence of a metal and reactive oxygen species selected to specifically cleave the protein into antigenic fragments with epitopes. The cleavage into fragments according to the invention is specific and reproducible, and is distinct from dissociation into subunits, modification of side chains, and protein degradation, all of which are common reaction pathways for proteins.

More particular examples according to the invention involve fragmenting autoantigens by adding iron at a concentration of between about 1μM and about 500μM, preferably between about 10μM and about 100μM. The method may include adding ascorbate, preferably in a concentration of between 1.7mM and 17mM. The autoantigens may be scleroderma autoantigens. In particular, topoisomerase I can be fragmented into a major fragment of 95kDa, and several minor, discrete species migrating between 65kDa and 90kDa. The large subunit of RNA polymerase II (a protein doublet of 220 and 240kDa) generated major species of 190, 160 and 140kDa, as well as several minor species of 200, 180, 170 and 130kDa. Upstream binding factor (UBF/NOR90) (a doublet of 90 and 100kDa) yielded fragments between 70kDa and 90kDa. The 70kDa protein component of the U1 small nuclear ribonucleoprotein (U1-70kDa) in response to Fe generated fragments that migrated between 33 and 38kDa.

Alternatively, suitable methods may involve a Cu-catalyzed oxidation system capable of fragmenting topoisomerase I, RNA polymerase II large subunit, and U1-70kDa, using about 10μM to about 500μM Cu, preferably about 20μM to about 100μM; and about 0.1mM to about 5mM H_2O_2, preferably about 1mM to about 2mM, to initiate oxygen radical production. The reaction may take place *in vitro*, in cellular lysates, in cell culture, or *in vivo*. With intact cells, fragmentation may be

accomplished by prolonged incubation with concentrations of extracellular Cu that are unable to induce protein fragmentation in cell lysates.

Another embodiment of the invention is a screening assay for a drug with anti-scleroderma or other effectiveness against another auto immune disease activity, comprising:

(i) establishing a control system comprising combining a self-tolerated autoantigen with iron or copper and reactive oxygen species, fragmenting the autoantigen, thereby producing a decrypted autoantigenic fragment, and detecting the decrypted fragment with an antibody to the fragment;

(ii) establishing a test system comprising adding a drug to the control system and determining whether the drug reduces the producing of a decrypted autoantigenic fragment; and

(iii) comparing the results of the control system and the test system to determine whether a particular drug is effective against the disease.

Related detection assays generally involve steps associated with detection of metal-catalyzed oxidative proteolysis in suitable cells or cell lysates. For example, one such assay generally includes the following steps 1) through 4):

1) culturing a population of suitable cells in medium comprising one or more Fenton metals in an amount sufficient to support metal-catalyzed oxidative proteolysis;

2) adding a known or candidate proteolytic inhibitor compound to the medium;

3) measuring production of autoantigenic and immunogenic protein fragments by the cells; and

4) determining the effect of the known or candidate proteolytic inhibitor compound on the production of the protein fragments.

A screening assay of the present invention can effectively measure the capacity of the known or candidate inhibitor compound to decrease production of the autoantigenic and immunogenic protein fragments. Measurements conducted in accord with the screening assays involve, e.g., quantitation of protein fragments by a suitable protein fragment detection assay such as those specified immunological assays described below. A suitable assay can be conducted with nearly any

population of cells capable of specifically cleaving autoantigens by the metal-catalyzed oxidative proteolysis reactions. Specific examples of suitable cells include immortalized cells such as HeLa cells and certain epithelial cells such as specified keratinocytes described in the examples that follow. Certain primary cells are also suitable for use in the assays.

Screening assays according to the invention can be adapted in accordance with intended use. In particular, nearly any disclosed screening assay can be modified as desired to detect compounds capable of inhibiting metal-catalyzed oxidative proteolysis of autoantigens. For example, instead of adding one or more Fenton metals to the cells as outlined in step 1) above, a lysate can be prepared from the cells and one or more than one of the Fenton metals can be added to the lysate in an amount sufficient to support metal-catalyzed oxidative proteolysis. Addition of the metal can be performed before, during or after administration of the known or candidate inhibitor compound specified in step 2), above. In most cases however, it will be preferred to add the known or inhibitor compound prior to adding the metal to the lysate.

A known or candidate inhibitor compound tested in any of the disclosed assays can be employed as a sole active agent or in combination with other agents including other known inhibitors of metal-catalyzed oxidative proteolysis to be tested, particularly those proteolytic reactions accompanied by Fenton or Haber-Weiss type chemical reactions. In most cases, the *in vitro* assays are performed with a suitable control assay usually including the same test conditions as in the steps and discussion above, but without adding the known or candidate inhibitor compound to the medium or cell lysate. In these instances, a candidate inhibitor compound can be identified as exhibiting a desired activity by providing at least about 10% greater inhibitory activity relative to the control; more preferably at least about 20% greater activity relative to the control assay; and still more preferably at least about 30%, 40%, 50%, 60%, 70%, 80%, 100%, 150%, or 200% greater activity relative to the control.

In particular, the inhibitory activity can be measured by a variety of methods including quantitation of autoantigenic and immunogenic protein fragments produced from autoantigens. Alternatively, inhibitory activity can be measured by monitoring production of ROS according to conventional techniques.

Further provided by the present invention are *in vitro* methods for reducing fragmentation of autoantigens comprising administering agents to reduce O_2 levels. Another method of reducing fragmentation is to reduce the availability of one or more Fenton metals such as iron or copper, preferably by adding a chelator such as 1mM desferroxamine, 1mM D-penicillamine, or 1mM EDTA. For copper, the chelator may be 200μM bathocuproine disulfonate (a Cu(I)-specific chelator). Adding zinc in a concentration up to about 300μM also inhibits fragmentation, as does chemical modification of the accessible carboxylates by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) prior to metal-mediated oxidation. Other metals and competing compounds may be used to inhibit fragmentation.

Additional aspects of the present invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of an immunoblot illustrating several scleroderma autoantigens uniquely fragmented by Fe/ascorbate or Cu/H_2O_2 oxidation reactions. HeLa lysates were prepared as described in the Methods section, and metal-catalyzed oxidation reactions were performed by adding the following: no additions (lane 1), 1.7mM ascorbate (lane 2), 1mM H_2O_2 (lane 3), 100μM Fe(II)SO_4 (lane 4), 100μM Fe(II)SO_4 + 1.7mM ascorbate (lane 5), 100μM Fe(II)SO_4 + 1mM H_2O_2 (lane 6), 100μM Cu(II)SO_4 (lane 7), 100μM Cu(II)SO_4 + 1.7mM ascorbate (lane 8) and 100μM Cu(II)SO_4 + 1mM H_2O_2 (lane 8). Samples were immunoblotted with the sera denoted on the left side of each panel. Equal amounts of protein were electrophoresed in the lanes of each panel. Migration positions of molecular weight standards are indicated on the right.

Figures 2A and 2B are representations of polyacrylamide gels showing that fragmentation of scleroderma autoantigens by oxidation is a highly specific event. In Figures. 2A and 2B equal protein amounts of control HeLa lysates (lanes 1 & 3), and HeLa lysates incubated with 100μM Fe + 1.7mM ascorbate (lanes 2 & 4) were electrophoresed.

The gel in Figure 2A was stained with coomassie blue to visualize the total protein profile of each sample. Oxidation catalyzed by Fe/ascorbate caused no striking alterations in the coomassie pattern, with the exception of the decreased

staining of an ~8kDa protein (arrow). Migration positions of the molecular weight standards are indicated to the right of lane 2.

In Figure 2B, electrophoresed proteins were immunoblotted with patient sera recognizing NuMA, La and 52kDa and 60kDa Ro, or a monoclonal antibody to fodrin (Chemicon International, Temecula, CA). None of these antigens were fragmented by Fe/ascorbate oxidation reactions (lanes 3 & 4).

Figures 3A, 3B, and 3C are representations of immunoblots showing that metal chelators, zinc or EDAC strongly inhibit or abolish fragmentation of the scleroderma autoantigens induced by both Fe/ascorbate and Cu/H_2O_2. The following additions were made to equal protein amounts of HeLa cell lysate, prior to immunoblotting with the indicated antibodies:

Figure 3A, none (lane 1); 100μM Fe + 1.7mM ascorbate (lanes 2, 4, 6, 8); 100μM Cu + 1mM H_2O_2 (lanes 3, 5, 7, 9); 1mM desferroxamine (lanes 4 & 5); 200μM bathocuproine disulfonate (lanes 6 & 7) or 1mM D-penicillamine (lanes 8 & 9). The band migrating at 66kDa in the UBF/NOR90 immunoblot is nonspecific.

Figure 3B, Increasing concentrations of $ZnCl_2$ (0-300μM) were added to HeLa lysates prior to addition of 100μM Fe + 1.7mM ascorbate. The IC_{50} value for inhibition of Fe/ascorbate-induced fragmentation of topoisomerase I or RNA polymerase II large subunit was 30-50μM.

Figure 3C, HeLa lysates were incubated in the absence (-) or presence (+) of 5mM EDAC, prior to adding 100μM Fe and 1.7mM ascorbate. In Figures 3A-C, equal protein amounts were loaded in each lane. Results are representative of two to six separate experiments.

Figure 4 is a representation of an immunoblot illustrating that topoisomerase I is fragmented in intact keratinocytes chronically exposed to 20μM Cu. Confluent monolayers of human foreskin keratinocytes were cultured for 18 hours (lanes 3 & 4) or 2 hours (lanes 5 & 6) in keratinocyte growth medium supplemented with 20μM $CuSO_4$. Control cultures were maintained in the absence of added Cu (lanes 1 & 2). Before harvesting the cells, 2mM H_2O_2 was added to some of the cultures (lanes 2, 4 & 6) but not others (lanes 1,3 & 5) for 30 minutes. Cells were subsequently harvested and immunoblotted with anti-topoisomerase I serum as described in Figure 1. Equal

amounts of protein were electrophoresed in each lane. Results are representative of those obtained in three separate experiments.

Figures 5A and 5B are photomicrographs showing that metal ions and topoisomerase I are concentrated in punctate intranucleolar structures.

In Figure 5A, HeLa cells were fixed in 1% lead acetate, and subsequently stained with 1% ammonium sulfide. Bright field microscopy demonstrates intense staining of punctate intranucleolar structures (nucleolini).

In Figure 5B, HeLa cells were stained with a monospecific human serum recognizing topoisomerase I and FITC-goat anti-human IgG, and were examined by confocal fluorescence microscopy. Diffuse nuclear staining as well as punctate intranucleolar structures (nucleolini) are seen. Similar results were obtained with 6 other monospecific topoisomerase I sera. Nucleolini were never stained when similar experiments were performed using sera obtained from healthy individuals, or from Ro/La-positive lupus patients. Bar, 10μM.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention features therapeutic methods for treatment or prevention of autoimmune disorders modulated by metal-catalyzed oxidative proteolysis of autoantigens. Such treatment methods generally include administering a therapeutically effective amount of a composition of the invention to a subject, preferably a patient in need of such treatment.

More particularly, therapeutic methods of the invention comprise administration of a therapeutically effective amount of a proteolytic inhibitor compound to a subject in need of such treatment, such as a mammal, and particularly a primate such as a human.

Additional treatment methods of the invention include administration of an effective amount of one or more autoantigenic and immunogenic protein fragment as defined herein to a subject, particularly a mammal such as a human in need of such treatment for an indication disclosed herein. Preferred fragments are those that are capable of substantially or completely tolerizing the mammal to the autoantigen or protein fragment as determined by standard immunological assays such as those disclosed below. Typical subjects include mammals suffering from or susceptible to an autoimmune disorder such as those specified below. Examples of suitable

immunological assays include Western immunoblot assays involving detection of patient autoantibodies against specified autoantigens.

The phrase "metal-catalyzed oxidative proteolysis" is used herein to denote specific cleavage of at least one autoantigen by a Fenton or Haber-Weiss type chemical reaction. The proteolysis can be achieved *in vitro* or *in vivo*. Typically, the metal will be iron or copper. See the examples which follow.

By the term "specific cleavage" or similar term is meant site-specific hydrolysis of an autoantigen or protein fragment disclosed herein by metal-catalyzed oxidative proteolysis. Site specific cleavage of the autoantigen or protein fragment can be readily detected and quantified if desired by, e.g., Western immunoblotting, ELISA, radioimmunoassay (RIA), antigen capture type assays, multiple-antibody sandwich type assays or other suitable immunoassays known in the field.

A variety of proteolytic inhibitor compounds can be employed in the present treatment methods. Simple testing, e.g., in a standard *in vitro* assay as defined above, can readily identify suitable proteolytic inhibitor compounds. Preferred proteolytic inhibitor compounds include those capable of eliminating or reducing metal-catalyzed oxidative proteolysis. Specific examples of such compounds include zinc and certain metal chelators capable of forming coordination complexes with metals and particularly with iron and copper. Generally preferred for use in the treatment methods of the invention are compounds such as zinc and metal chelators such as desterroxamine, D-penicillamine, ethylenediamine tetraacetic acid (EDTA), bathocuproine disulfonate, or related compounds.

In the therapeutic methods of the invention, a treatment compound can be administered to a subject by one or a combination of strategies. For example, a proteolytic inhibitor compound can be administered as a prophylactic to prevent the onset of or reduce the severity of a targeted condition. Alternatively, a proteolytic inhibitor compound can be administered during the course of a targeted condition.

A treatment compound can include one or more proteolytic inhibitor compounds or one or more autoantigenic protein fragments according to the invention. The treatment compound can be administered to a subject, either alone or in combination with one or more other therapeutic compounds (agents), as a pharmaceutical composition in mixture with conventional excipient, i.e.

pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds and are not deleterious to the recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the active compounds.

Such compositions may be prepared for use in parenteral administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; intranasally, particularly in the form of powders, nasal drops, or aerosols; vaginally; topically e.g. in the form of a cream; rectally e.g. as a suppository; etc.

The pharmaceutical agents may be conveniently administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical arts, e.g., as described in *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980). Formulations for parenteral administration may contain as common excipients such as sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be useful excipients to control the release of certain proteolytic inhibitor compounds.

Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally.

Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration. Other delivery systems will administer the therapeutic agent(s) directly at a selected body site, e.g. by use of stents.

A proteolytic inhibitor compound or autoantigenic fragment can be employed in the present treatment methods as the sole active pharmaceutical agent or can be used in combination with other active ingredients, e.g., one or more recognized immunomodulatory, anti-inflammatory or analgesic compounds such as cyclosporin, cortisol, or aspirin, respectively.

The concentration of one or more treatment compounds in a therapeutic composition will vary depending upon a number of factors, including the dosage of the compound to be administered, the chemical characteristics (e.g., hydrophobicity) of the composition employed, and the intended mode and route of administration. In general terms, one or more than one of the compounds may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v of a compound for parenteral administration.

In cases where it is desired to administer an autoantigenic and immunogenic protein fragment in accord with the invention, the fragment may be chemically modified, e.g., by modification of C- and/or N-terminii according to conventional methods to increase half-life.

It will be appreciated that the actual preferred amounts of active compounds used in a given therapy will vary according to e.g. the specific compound being utilized, the particular composition formulated, the mode of administration and characteristics of the subject, e.g. the species, sex, weight, general health and age of the subject. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines. Suitable dose ranges may include from about 1μg/kg to about 100mg/kg of body weight per day.

Therapeutic compounds of the invention are suitably administered in a protonated and water-soluble form, e.g., as a pharmaceutically acceptable salt, typically an acid addition salt such as an inorganic acid addition salt, e.g., a hydrochloride, sulfate, or phosphate salt, or as an organic acid addition salt such as an

acetate, maleate, fumarate, tartrate, or citrate salt. Pharmaceutically acceptable salts of therapeutic compounds of the invention also can include metal salts, particularly alkali metal salts such as a sodium salt or potassium salt; alkaline earth metal salts such as a magnesium or calcium salt; ammonium salts such an ammonium or tetramethyl ammonium salt; or an amino acid addition salts such as a lysine, glycine, or phenylalanine salt.

Additionally contemplated therapeutic compounds include novel chelators, agents that bind to metal-binding sites, and peptides for tolerizing strategies.

Several particular therapeutic methods are contemplated. For example, identification of agents which decrease fragmentation of antigens *in vitro* and in perturbed tissues of patients with autoimmune diseases, including scleroderma. Two broad groups of agents are envisioned:

a. those that chelate metals, particularly those capable of catalyzing a Fenton reaction.

b. those that compete for the binding site of these metals in nucleolini, and thereby prevent Fenton chemistry from being catalyzed at that site.

While specific fragmentation may break tolerance to self-proteins when presentation occurs under pro-immune conditions, delivery of these fragments under normal conditions generally results in their being tolerized. Since the immune response in scleroderma is restricted to very few molecules, identification of these fragments and their use as tolerizing agents may be of great significance in prevention of the disease in at-risk populations.

The compositions of the present invention, i.e., antigenic protein fragments, and inhibitors of protein fragmentation such as chelators and compounds that compete with the metal catalysts, may be made into pharmaceutical compositions with appropriate pharmaceutically acceptable carriers or diluents. If appropriate, pharmaceutical compositions may be formulated into preparations including, but not limited to, solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, and aerosols, or other suitable administration formats. A pharmaceutically-acceptable form should be employed which does not ineffectuate the compositions of the present invention. In pharmaceutical dosage forms, the compositions may be used alone or in

appropriate association, as well as in combination with, other pharmaceutically-active compounds.

Accordingly, the pharmaceutical compositions of the present invention can be delivered via various routes and to various sites in an animal body to achieve a particular effect. Local or system delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation, or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, peritoneal, subcutaneous intradermal, as well as topical administration.

Preferably, for tolerization, the antigenic protein fragments are administered intravenously without adjuvants. The inhibitors of protein fragmentation such as chelators are effectively administered systemically, with the resulting effect in the intracellular environment.

The "therapeutically effective amount" of the composition is such as to produce the desired effect in a host which can be monitored using several end-points known to those skilled in the art. For example, one desired effect might comprise tolerization towards self-antigens, and another would inhibition of the autoimmune disease. Such effects could be monitored in terms of a therapeutic effect, e.g., alleviation of some symptom associated with the disease being treated, or further evidence of lack of auto immunity, or particularized assays. These methods described are by no means all-inclusive, and further methods to suit the specific application will be parent to the ordinary skilled artisan.

Furthermore, the amounts of each active agent included in the compositions employed the examples described herein provide general guidance of the range of each component to utilized by the practitioner upon optimizing the method of the present invention for practice either *in vitro* or *in vivo*. Moreover, such ranges by no means preclude use of a higher or lower amount of a component, as might be warranted in a particular application. For example, the actual dose and schedule may vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or depending on interindividual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts may vary for *in vitro* applications. One skilled in the art can easily make any necessary adjustments in accordance with the necessities of the particular situation.

As noted above, the invention also relates to methods for inducing fragmentation of autoantigens as initiators of autoimmunity. The process reveals immunocryptic epitopes in self-antigens which may initiate the autoimmune response.

In particular, scleroderma is a systemic sclerosis which may affect a patient's entire body. Its symptoms may be related to a vascular lesion, ischemia reperfusion, which is associated with an autoimmune response. The reversible ischemia-reperfusion which characterizes scleroderma is associated with reactive oxygen species which can induce autoantigen fragmentation. Several of the autoantigens targeted in diffuse scleroderma are uniquely susceptible to cleavage by reactive oxygen species, in a metal-dependent manner. multiple features of the fragmentation reaction and its inhibition indicate that these autoantigens possess metal-binding sites, which focus metal-catalyzed oxidation reactions (and consequent fragmentation) to specific regions of the antigens.

The methods and products of the invention provide unique antigenic protein fragments. They are antigenic in the sense that they bind with antibodies, but also in the sense that they are immunogenic, and can trigger the autoantibody response in autoimmune diseases. Immunogenicity is a property of substances (immunogens or antigens) that can induce a detectable immune response, which can be humoral, cellular, or both.

The therapeutic methods of the invention include altering concentrations of metals. Abnormal metal status is a potential pathogenic principle in this disease, and can also be used as a diagnostic or therapeutic tool. The fragments can be used as tolerizing agents in therapy of autoimmune diseases.

The term "treatment" is intended to encompass administration of compounds according to the invention propylactically to prevent or suppress an undesired condition, and therapeutic administration to eliminate or reduce the extent or symptoms of the condition. Treatment according to the invention may be for a human or an animal having a disease in need of such treatment, or it may include application *in vitro* to a cell culture or extracellular media. Treatment may be by systemic administration to a patient or locally to an affected site.

This invention relates to the mechanism involved in the breakdown of self tolerance to induce autoimmune responses to specific autoantigens, a subject that has

been intensely studied over the years. Tolerance traditionally is defined as a state of specific immunological unresponsiveness to an antigen, which occurs when the immune system has had prior contact with that antigen, or operationally is defined as a specific, antigen-induced, depression of an immune response. The development of tolerance to an endogenous or a self antigen (autoantigen) is termed "natural immunological" or "self" tolerance, whereas immunological non-responsiveness known as "acquired immunological" tolerance arises upon exposure to an exogenous or a foreign antigen. The ability of the immune system to respond positively to foreign antigens while negatively responding to self-antigens has been a key paradox in the study of immunology. The relationship between self tolerance and induced tolerance continues to be debated. Unraveling the pathogenesis of autoimmune diseases is a challenging quest, but provides opportunities for specific diagnostic evaluations and therapeutic interventions not previously available.

Autoimmunity is the process of developing an immune response to self antigens. Autoimmune diseases may involve the generation of autoantibodies and/or the development of autoreactive T lymphocytes. Autoimmune diseases comprise a varied list of diseases, which possess a myriad of clinical manifestations. Different autoantigens and pathogens are associated with specific autoimmune diseases. Autoantigens may involve intracellular antigens, such as NA, small nuclear ribonucleoproteins, and intracellular proteins; membrane receptors, such as thyrotropin, acetylcholine receptor, insulin receptor; cell membranes, such as red blood cells, lymphocytes, neutrophils, platelets, muscle, and islet cells; plasma proteins, such as immunoglobulins, complement, clotting factors; and hormones, such as insulin, thyroid hormones, glucagon, and intrinsic factor.

The term "autoimmune disease" as applicable to the present invention includes scleroderma, multiple sclerosis, myasthenia gravis, polymyositis, graft-versus-host disease, graft rejection, raves disease, Addison's disease, autoimmune uveoretinitis, pemphigus vulgaris, primary biliary cirrhosis, rheumatoid arthritis, psoriasis, atopic dermatitis, osteoarthritis, Hashimoto's thyroiditis, primary myxoedema, thyrotoxicosis, pernicious anemia, autoimmune atrophic gastritis, insulin-dependent diabetes mellitus, Goodpasture's syndrome, pemphigoid, sympathetic opthalmia, phacogenic uveitis, autoimmune haemolytic anemia, idiopathic thrombocytopenic

purpura, idiopathic leucopenia, active chronic hepatitis, cryptogenic cirrhosis, ulceratiye colitis, Sjogren's syndrome, dermatomyositis, mixed connective tissue disease, discoid lupus erythematosus, systemic lupus erythematosus, and other conditions known in the art.

The methods and materials of the invention apply best to those autoimmune diseases in which autoantigens are cleaved to antigenic fragments by oxidative proteolysis such as Fenton chemistry or otherwise. These include rheumatoid arthritis, pulmonary fibrosis, myositis, primary biliary cirrhosis, and autoimmune thyroid disease. Experimental work confirms the suitability of oxidative proteolysis for these diseases. Other diseases, such as lupus and Sjogren's syndrome are believed to involve enzymatic proteolysis in their natural progression and so are less susceptible to the techniques of this invention.

Diagnosis of an autoimmune disease according to the invention may include identification of antibodies to the revealed epitopes of the autoantigen fragments produced by metal-mediated oxidative proteolysis *in vivo* or *in vitro*.

In particular, autoantigenic and immunogenic protein fragments can be made in accord with the invention and then used in a conventional immunological assay to identify autoantibodies in patient sera. Specific binding between the sera and one or more of the protein fragments is indicative of the autoimmune disease. See the examples which follow.

As noted, antibodies of the invention are particularly useful as diagnostic agents for detecting autoantigenic and immunogenic protein fragments in biological samples such as those obtained from a subject such as a mammal and particularly a primate such as a human patient. The antibodies can be prepared by recognized immunological techniques and are typically generated to a purified sample of a desired protein fragment. A substantially pure autoantigenic and immunogenic protein fragment produced by a method of the present invention is particularly preferred. Such antibodies also can be generated from an immunogenic peptide that comprises one or more cryptic epitopes of the protein fragment. As noted above, such cryptic epitopes are typically not exhibited by autoantigens. Monoclonal antibodies are generally preferred, although polyclonal antibodies also can be employed in the present methods.

More particularly, antibodies can be prepared by immunizing a mammal with a purified sample of a desired autoantigenic and immunogenic protein fragment or an immunogenic peptide as discussed above, alone or complexed with a carrier. Suitable mammals include typical laboratory animals such as sheep, goats, rabbits, guinea pigs, rats and mice. Rats and mice, especially mice, are preferred for obtaining monoclonal antibodies. The antigen can be administered to the mammal by any of a number of suitable routes such as subcutaneous, intraperitoneal, intravenous, intramuscular or intracutaneous injection. The optimal immunizing interval, immunizing dose, etc. can vary within relatively wide ranges and can be determined empirically based on this disclosure. Typical procedures involve injection of the antigen several times over a number of months. Antibodies are collected from serum of the immunized animal by standard techniques and screened to find specified antibodies. Monoclonal antibodies can be produced in cells which produce antibodies and those cells used to generate monoclonal antibodies by using standard fusion techniques for forming hybridoma cells. See G. Kohler, et al., *Nature*, **256**:456 (1975). Typically this involves fusing an antibody producing cell with an immortal cell line such as a myeloma cell to produce the hybrid cell. Alternatively, monoclonal antibodies can be produced from cells by the method of Huse, et al., *Science*, **256**:1275 (1989).

One suitable protocol provides for intraperitoneal immunization of a mouse with a composition comprising a substantially pure autoantigenic and immunogenic fragment produced from topoisomerase I (see Example 1 below) over a period of about two to seven months. Spleen cells then can be removed from the immunized mouse. Sera from the immunized mouse is assayed for titers of antibodies specific for the protein fragment prior to excision of spleen cells. The excised mouse spleen cells are then fused to an appropriate homogenic or heterogenic (preferably homogenic) lymphoid cell line having a marker such as hypoxanthine-guanine phosphoribosyltransferase deficiency ($HGPRT^-$) or thymidine kinase deficiency (TK^-). Preferably a myeloma cell is employed as the lymphoid cell line. Myeloma cells and spleen cells are mixed together, e.g. at a ratio of about 1 to 4 myeloma cells to spleen cells. The cells can be fused by the polyethylene glycol (PEG) method. See G. Kohler, et al., *Nature, supra*. The thus cloned hybridoma is grown in a culture

medium, e.g. RPMI-1640. See G. E. More, et al., *Journal of American Medical Association*, **199**:549 (1967). Hybridomas, grown after the fusion procedure, are screened such as by radioimmunoassay or enzyme immunoassay for secretion of antibodies that bind specifically to the purified protein fragment. Preferably an ELISA is employed for the screen. Hybridomas that show positive results upon such screening can be expanded and cloned by limiting dilution method. Further screens are preferably performed to select antibodies that can bind to the protein fragment in solution as well as in a human fluid sample. The isolated antibodies can be further purified by any suitable immunological technique including affinity chromatography.

For some applications, it may be desirable to produce chimeric antibody derivatives, e.g. antibody molecules that combine a non-human animal variable region and a human constant region, to thereby render the antibodies less immunogenic in a human subject than the corresponding non-chimeric antibody. A variety of types of such chimeric antibodies can be prepared, including e.g. by producing human variable region chimeras, in which parts of the variable regions, especially conserved regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. See also discussions of humanized chimeric antibodies and methods of producing same in S. Morrison, *Science*, **229**:1202-1207 (1985); Oi et al., *BioTechniques*, 4:214 (1986); Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, **80**:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (9183); Olsson et al., *Meth. Enzymol.*, **9**:3-16 (1982).

The molecular weight of the antibodies of the invention will have a molecular weight of between approximately 20 to 150kDa. Such molecular weights can be readily are determined by molecular sizing methods such as SDS-PAGE gel electrophoresis followed by protein staining or Western blot analysis.

"Antibody of the invention" or other similar term refers to whole immunoglobulin as well immunologically active fragments which bind a desired autoantigenic and immunogenic protein fragment. The immunoglobulins and immunologically active fragments thereof include an antibody binding site (i.e., a cryptic epitope capable of specifically binding the antibody). Exemplary antibody fragments include, for example, Fab, F(v), Fab', $F(ab')_2$ fragments, "half molecules" derived by reducing the disulfide bonds of immunoglobulins, single chain

immunoglobulins, or other suitable antigen binding fragments (see e.g., Bird et al., *Science*, pp. 242-424 (1988); Huston et al., *PNAS, (USA)*, 85:5879 (1988); Webber et al., *Mol. Immunol.*, 32:249 (1995)). The antibody or immunologically active fragment thereof may be of animal (e.g., a rodent such as a mouse or a rat), or chimeric form (see Morrison et al., *PNAS*, 81:6851 (1984); Jones et al., *Nature*, pp. 321, 522 (1986)). Single chain antibodies of the invention can be preferred.

By the term "specific binding" as it relates to an antibody of the invention is meant an antibody which is capable of forming an immune complex with an autoantigenic and immunogenic protein fragment as described herein. Preferably, the antibody is capable of specifically binding cryptic epitopes but is not capable of specifically binding the autoantigen that gave rise to the peptide fragment as determined by, e.g., Western blotting, ELISA, RIA, or other suitable protein binding assays known in the field.

It is noted that some antibodies and reactive sera disclosed herein are capable of binding a specified autoantigen and autoantigenic and immunogenic protein fragments produced from same. Such antibodies and reactive sera are useful for a variety of applications in accord including detecting the protein fragments. See e.g., Fig. 1.

Preferred antibodies of the invention are typically monoclonal and specifically bind a cryptic epitope predominant to a desired autoantigenic and immunogenic protein fragment produced as disclosed herein. Indeed, preferred antibodies of the invention bind to the protein fragment at least about 2, 5, 10, 25, 50, 100 or more times greater than the binding affinity to the autoantigen from which the protein fragment was produced. Preferred antibodies of the invention do not substantially bind the autoantigen as determined by any suitable immunological assay described herein.

As discussed above, antibodies of the invention can be used to detect presence or to quantitate if desired, one or more autoantigenic and immunogenic protein fragments produced by metal-catalyzed oxidative proteolysis. Preferred detection methods include suitable immunological assays such as a Western immunoblot. Particularly preferred detection methods include Western immunoblotting followed

by conventional densitometery to quantitate presence of the protein fragments in suitable control and experimental samples.

Antibodies of the invention can also be employed in accord with standard immunological practice either *in vivo* or in situ to detect production of autoantigenic and immunogenic protein fragments in cells, tissues and organs. For example, such antibodies are particularly useful in the subcellular identification of the protein fragments in nucleolini.

A "polypeptide" refers to any polymer consisting essentially of any of the 20 amino acids regardless of its size. Although the term "protein" is often used in reference to relatively large proteins, and "peptide" is often used in reference to small polypeptides, use of these terms in the field often overlaps. The term "polypeptide" refers generally to proteins, polypeptides, and peptides unless otherwise noted.

The autoantigenic and immunogenic protein fragments of the present invention can be separated and purified by appropriate combination of known techniques. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electrical charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatograph, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatograph and methods utilizing a difference in isoelectric point, such as isoelectric focusing electrophoresis, metal affinity columns such as Ni-NTA. See generally Sambrook et al., *Molecular Cloning: A Laboratory Manual.* (2nd ed. (1989); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1989) for disclosure relating to these and other conventional methods for purifying proteins and protein fragments.

It is preferred that the autoantigenic and immunogenic protein fragments of the present invention be substantially pure. That is, the protein fragments have been isolated from cell substituents that naturally accompany it so that the fragments are present preferably in at least 80% or 90% to 95% homogeneity (w/w). Suitable protein fragments having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified

the fragments should be substantially free of contaminants for therapeutic applications. Once purified partially or to substantial purity, the protein fragments can be used therapeutically, or in performing *in vitro* or *in vivo* assays as disclosed herein. Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

As noted above, various assays in accord with the invention can be used to detect or test known or candidate compounds for therapeutic capacity to inhibit metal-catalyzed oxidative proteolysis of a desired autoantigen, e.g., topoisomerase I. Exemplary assays generally involve monitoring Fenton or Haber-Weiss type chemical reactions to produce one or more autoantigenic and immunogenic protein fragments from the autoantigen. One preferred assay is a standard metal-catalyzed Fenton reaction involving the following steps a) through e):

a) preparing a lysate from about 1 X 10^6 or less confluent cultured cells such as HeLa cells and adding to the cell lysate: 1) a known or candidate compound in an amount between about 0.1 μM to 100μM and 2) at least one Fenton metal salt such as ferrous sulfate in an amount between about 0.1μM to 100μM, preferably one such Fenton metal salt;

b) incubating the HeLa cell lysate for a time sufficient to allow the metal-catalyzed oxidative proteolysis, e.g., for about 30 minutes, and typically followed by centrifugation to remove debris;

c) separating the lysate, e.g., by SDS-PAGE gel electrophoresis, and transferring the separated lysate to a solid support suitable for performing a standard Western immunoblot, e.g., nitrocellulose;

d) contacting the solid support with an antibody (or reactive sera comprising same) under conditions sufficient to form an immune complex between at least one autoantigenic and immunogenic protein fragment and the antibody; and

e) detecting formation of the immune complex on the solid support.

Preferably the antibody is an autoimmune antibody (autoantibody) capable of specifically binding autoantigenic fragments of specified nucleic acid binding proteins such as topoisomerase I. The antibody or reactive sera may also bind other autoantigens such as those described herein. Methods for preparing monospecific

patient autoimmune sera to topoisomerase I (and other autoantigens) are known in the field and are discussed below.

Reference herein to a "standard metal-catalyzed Fenton reaction" or other similar phrase includes one or more of the steps a) through e) above. It will be apparent from the discussion and examples that follow that the standard Fenton reaction can be readily modified to suit intended use. For example, the iron salt in the standard Fenton reaction can be substituted with one or more copper salts as described below. Other modifications include adding specified amounts of ascorbate or hydrogen peroxide. Additionally, the standard Fenton reaction described above can accomadate use of antibodies (or reactive sera) against a variety of autoantigens, e.g., RNA polymerase II large subunit, UBF/NOR90, or U1-70kDa. See the examples which follow.

All documents mentioned herein are incorporated by reference herein in their entirety.

The present invention is further illustrated by the following non-limiting examples and discussion.

General Comments

The following materials and methods (numbered 1-5 below) were used in Examples below.

1. Sera. After obtaining informed consent, human autoimmune sera were collected from 60 patients with diffuse scleroderma, and their reactivity with saline soluble extracts of rabbit thymus was determined by Ouchterlony immunodiffusion using standard reference serum to topoisomerase I. The sera were further screened by immunoblotting against control HeLa cell lysates, using reference antibodies to NOR90/UBF, U1-70kDa, topoisomerase I, and RNA polymerase I, II, and III as standards. Of the RNA polymerases, only the large subunit of RNA polymerase II was recognized by immunoblotting. Other autoimmune sera recognizing lupus autoantigens have been described previously (L. Casciola-Rosen et al., *J. Exp. Med.*, **179**:1317-1330 (1994); Casciola-Rosen, et al. *J. Biol. Chem.* **269**: 30757-30760 (1994); Casciola-Rosen, et al. *J. Exp. Med.* **183**: 1957-1964 (1996)).

2. Metal-catalyzed oxidation reactions. Confluent HeLa monolayers were washed twice with KRB (20mM Hepes pH 7.4, 127mM NaCl, 5.5mM KCl, l0mM

dextrose, 1mM $CaCl_2$ and 2mM $MgSO_4$), and then lysed in a buffer containing 20mM Hepes pH 7.4, 1% Nonidet P40, 150mM NaCl and the following protease inhibitors: leupeptin, pepstatin A, chymostatin, antipain and PMSF. Equal lysate aliquots were pipetted into microfuge tubes, prior to adding metal, H_2O_2 and/or ascorbate. Samples were incubated at 25°C for 30 minutes, with vortex mg every 5 minutes. Reactions were terminated by adding 10mM EDTA, SDS gel buffer and boiling for 3 minutes. Equal protein amounts of each sample were electrophoresed on 10% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose and immunoblotted with patient sera monospecific for topoisomerase I, RNA polymerase II large subunit, UBF/NOR90 or U1-70kDa. Blotted proteins were visualized using the Supersignal CL-HRP substrate system (Pierce, Rockford, IL) according to the manufacturer's directions. Primary antibodies were used at dilutions of 1:5,000-1:20,000, and autoradiogram exposure times ranged from 2 seconds-1 minute. Experiments were always performed using freshly-made lysate, and fresh stock solutions of Fe, Cu, H_2O_2 and ascorbate. Oxygen-depletion was accomplished by vigorously blowing O_2-free argon through the lysate for 5 minutes prior to addition of Fe and ascorbate. For these experiments, reactions on both control and O_2-depleted lysates were performed in sealed tubes (without vortexing) at 25°C for 30 minutes.

3. Metal chelator, EDAC and zinc competition experiments. Metal-catalyzed oxidation reactions were performed on HeLa lysates essentially as described above, with the following modifications. Experiments performed in the presence of 1mM desferroxamine, 200μM bathocuproine disulfonate or 1mM D-penicillamine were carried out by adding the chelators immediately prior to addition of the Fe or Cu. $ZnCl_2$ competition reactions, and those performed in the presence of 5mM EDAC, were executed by adding these reagents to HeLa lysates 5mM prior to adding Fe/ascorbate.

4. Intact Cell Experiments. Confluent monolayers (secondary passage) of human foreskin keratinocytes were cultured as described (L. Casciola-Rosen et al., *J. Exp. Med.*, **179**:1317-1330 (1994)). Experiments were initiated by adding keratinocyte growth medium supplemented with 20μM $CuSO_4$ to the cells for 2 hours or 18 hours. 2mM H_2O_2 was subsequently added to some of the dishes, and after a further 30 minutes incubation, the cells were harvested and immunoblotted.

5. Morphologic studies. Microscopy was performed on HeLa cells grown on No. 1 glass coverslips. For immunofluoresence, cells were fixed in 4% paraformaldehyde (5 minutes, 4°C), permeabilized in acetone (30 seconds, 4°C), and stained using a standard monospecific patient serum recognizing topoisomerase I (diluted 1:160 in PBS). Bound antibodies were visualized with FITC-conjugated goat anti-human F(ab')2 (Organon Tekitika Corp./Cappel, Durham, NC). Coverslips were mounted with Permafluor (Lipshaw, Pittsburgh, PA), and viewed on a scanning confocal microscope system (model MRC 600, Bio-Rad Laboratories). For metal staining, cells were fixed in filtered 1% lead acetate pH 5.5 (30 minutes, 37°C), washed extensively with H_2O, and stained with 1% ammonium sulfide (5 minutes, 25°C). After extensive washing with H_2O coverslips were mounted, viewed and photographed under bright field on a Zeiss Axiophot microscope.

Example 1- Scleroderma autoantigens are fragmented by metal-catalyzed oxidation reactions

Substantial evidence exists for the *in vitro* production of reactive oxygen species (including hydroxyl radical ($OH^{\cdot}$)) via the metal-catalyzed Fenton ($H_2O_2 + Fe^{2+}(Cu^{+}) \rightarrow Fe^{3+}(Cu^{2+}) + OH^{-} + OH$) and Haber-Weiss reactions ($O_2^{\cdot} + H_2O_2 \longrightarrow O_2 + OH^{-}$) (reviewed in (R. Youngman, *TIBS*, pp. 280-283 (1984); B. Halliwell et al., *Arch. Biochem. Biophys.*, **246**:501-514 (1986)). In initial studies, we added ferrous sulfate (Fe) to NP-40 lysates of HeLa cells to generate free radical species by Fenton chemistry (R. Levine et al., *Proc. Natl. Acad. Sci. USA*, **78**:2120-2124 (1981); R. Levine et al., *J. Biol. Chem.*, **258**:11828-11833 (1983); J. Creeth et al., *Biochem. J.*, **211**:323-332 (1983); B. Cooper et al., *Biochem. J.*, **228**:615-626 (1985)), and addressed whether several of the major autoantigens in diffuse scleroderma were fragmented (J. Shero et al., *Science*, **231**:737-740 (1986); J. Rodriguez-Sanchez et al., *J. Immunol.*, **139**:2579-2584 (1987); M. Hirakata et al., *J. Clin. Invest.*, **91**:2665-2672 (1993); E. Tan, *Cell*, **67**:841-842 (1991)). After incubating *in vitro* for 30 minutes in the absence of added metal, topoisomerase I remained stable, and migrated as a single 100kDa species (Figure 1, lane 1). In these metal-free conditions, addition of either ascorbate or H_2O_2 failed to generate any fragments (Figure 1, lanes 2 & 3). Addition of 100μM Fe induced the specific fragmentation of topoisomerase I, generating a major fragment of 95kDa, and several minor, discrete species migrating between

65kDa and 90kDa (Figure 1, lane 4). In the presence of 1.7mM ascorbate, generation of these fragments was enhanced (Figure 1, lane 5); the reaction went to completion (that is, total loss of the intact protein) when 17mM ascorbate was added. In contrast, 1mM H_2O_2 resulted in a slight but reproducible decrease in fragmentation when compared to Fe alone (Figure 1, lane 6 *vs* lane 4). Fe also induced specific fragmentation of three additional scleroderma autoantigens: the large subunit of RNA polymerase II, upstream binding factor (UBF/N0R90) and the 70kDa protein component of the U1 small nuclear ribonucleoprotein (U1-70kDa). Although the sensitivity of the different proteins to metal-induced fragmentation varied (e.g. the large subunit of RNA polymerase II was efficiently fragmented using <10μM Fe), cleavage was enhanced by the addition of ascorbate in all cases: (a) the large subunit of RNA polymerase II (a protein doublet of 220 and 240kDa) was specifically fragmented, generating major species of 190, 160 and 140kDa, as well as several minor species of 200, 180, 170 and 130kDa (Figure 1, lane 5); (b) Fragmentation of UBF/N0R90 (a doublet of 90 and 100kDa in control lysates) yielded a poorly resolved smear of fragments between 70kDa and 90kDa (Figure 1, lane 5); (c) U1-70kDa fragmentation in response to Fe generated a few discrete fragments that migrated between 33 and 38kDa (Figure 1, lane 5); this Fe-induced fragmentation was inefficient compared to that of the other autoantigens. Identical U1-70kDa fragments were also observed when [^{35}S]methionine-labeled U1-70kDa was generated by coupled *in vitro* transcription/translation, and then subjected to Fe/ascorbate treatment.

Some of the autoantigens that fragmented in response to Fe/ascorbate were also fragmented in a Cu-catalyzed oxidation system. Thus, specific fragments of topoisomerase I, RNA polymerase II large subunit and U1-70kDa were observed when 100μM Cu and 1mM H_2O_2 were used to initiate oxygen radical production (Figure 1, lanes 7-9). In contrast, no fragmentation of UBF/NOR90 was detected under these conditions (Figure 1, lanes 7-9). Several striking differences between the fragments generated by Cu/H_2O_2 and those generated by Fe/ascorbate were noted: (a) Addition of Cu alone did not result in autoantigen fragmentation, which was dependent on the addition of H_2O_2; (b) The fragments observed in response to Cu/H_2O_2 were distinct from those generated by Fe/ascorbate: Cu-induced

fragmentation of topoisomerase I produced a major species of 95kDa, but did not generate the other fragments detected in the Fe system (Figure 1, lanes 5 & 9). Cu-induced breakage of U1-70kDa generated a single product of 65kDa (not seen in the Fe system), and did not give rise to the 33-38kDa fragments typical of Fe-induced fragmentation (Figure 1, lanes 5 & 9). Incubation with Cu/H_2O_2 induced aggregation of the large subunit of RNA lymerase II (detected as immunoreactive protein species migrating >250kDa), and also gave rise to several distinct cleavage fragments migrating between 170 and 210kDa, which differed from the predominant Fe-induced fragments (Fig 1, lanes 5 & 9); (c) Topoisomerase I, the large subunit of RNA polymerase II, and UBF/NOR90 were more efficiently fragmented by Fe/ascorbate than Cu/H_2O_2. In contrast, fragmentation of U1-70kDa was more effectively induced with the latter oxidation system.

There is direct evidence that an aspartic acid-rich region in the large subunit of bacterial RNA polymerase (which chelates the active center Mg^{2+} ion (E. Zaychikov et al., *Science*, **273**:107-109 (1996)) can also bind Fe(II); under these circumstances, fragmentation of the protein occurs in the region of the metal-binding site in a reaction that requires generation of hydroxyl radicals (E. Zaychikov et al., *Science*, **273**:107-109 (1996)). Both metal-binding and cleavage were dependent upon the presence of an aspartic acid-rich motif (DFDGD) (E. Zaychikov et al., *Science*, **273**:107-109 (1996)), which is absolutely conserved in eukaryotic RNA polymerases I, II, and III (E. Zaychikov et al., *Science*, **273**:107-109 (1996)). It is of great interest that all three of the human RNA polymerases are autoantigens in scleroderma (M. Hirakata et al., *J. Clin. Invest.*, **91**:2665-2672 (1993)); the sizes of the fragments produced by metal-catalyzed oxidation of human RNA polymerase II large subunit (Figure 1) correspond well with those predicted to arise if cleavage occurred at the equivalent DFDGD site in this molecule.

Example 2- Fibrillarin is not fragmented by metal-catalyzed oxidation reactions

In contrast to the autoantigens described above in Example 1, fibrillarin, the major scleroderma ribonucleoprotein autoantigen, was not fragmented under identical reaction conditions.

Example 3- Autoantigen fragmentation reactions are specific

The results of Examples 1 and 2 above indicate that several of the antigens frequently targeted in patients with diffuse scleroderma are specifically fragmented in both of the metal-catalyzed oxidation systems. The specificity of these fragmentation reactions was confirmed in several different ways: (a) Except for the decreased staining of a protein of ~85kDa (Figure 2A, arrow), the coomassie blue staining of cell lysates incubated with Fe/ascorbate was not significantly different from control lysates (Figure 2A, lanes 1 & 2). The coomassie blue staining profile of control lysates and those treated with Cu/H_2O_2 were identical. These data are consistent with previous observations showing that although modification of proteins by metal-catalyzed reactions is frequent (K. Davies et al., *J. Biol. Chem.*, **262**:9895-9901 (1987); E. Stadtman, *Free Radic. Biol. Med.*, **9**:315-325 (1990); L. Fucci et al., *Proc. Natl. Acad. Sci. USA*, **80**:1521-1525 (1983)), fragmentation of these proteins is an extremely uncommon event (E. Stadtman, *Free Radic. Biol. Med.*, **9**:315-325 (1990)); (Figure 2B) Fragmentation reactions were absolutely dependent on metals capable of supporting Fenton chemistry (Fe, Cu); no autoantigen cleavages were observed upon addition of 100μM zinc, cobalt, mercury, magnesium, manganese, nickel, cadmium, or silver, either alone or in the presence of H_2O_2 or ascorbate; (c) None of the SLE autoantigens tested (including 52kDa and 60kDa Ro, La, and the Sm proteins), nor several other proteins proteolytically cleaved during apoptosis (including poly(ADP-ribose) polymerase, actin and the nuclear mitotic apparatus protein, NuMA) were fragmented in oxidation reactions containing Fe/ascorbate or Cu/H_2O_2 (see Figure 2B); (d) identical fragments were produced in more than 20 separate experiments, immunoblotted with 7 different topoisomerase I sera, and 3 different RNA polymerase II large subunit, U1-70kDa and UBF/N0R90 sera. Identical fragments were also generated in lysates of several different cell types, including human keratinocytes and human umbilical vein endothelial cells; and (e) fragmentation was inhibited under O_2-depleted conditions. These data confirm that the fragmentation observed is a site-specific oxygen and metal mediated proteolysis, as opposed to the more common phenomenon of enzymatic proteolysis.

Fragmentation of RNA polymerase II and topoisomerase I has been found to decrease in O_2-depleted lysates. O_2-depletion was performed as described Materials and Methods, prior to adding 100μM Fe and 1.7mM ascorbate. Equal protein

amounts were electrophoresed and immunoblotted with antibodies to RNA polymerase II and topoisomerase I.

Example 4- Metal chelators, zinc or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide inhibit the fragmentation of scleroderma autoantigens

The site-specific nature of metal-catalyzed oxidation reactions has previously indicated that these reactions are "caged" processes in which amino acid residues at metal-binding sites are specific targets of highly reactive free radical species generated at that site during a Fenton reaction (B. Halliwell et al., *Arch. Biochem. Biophys.*, **246**:501-514 (1986); E. Stadtman, *Ann. Rev. Biochem.*, **62**:797-821 (1993); T. Rana et al., *J. Am. Chem. Soc.*, **112**:2457-2458 (1990)). Sequestration of metals by chelators in the bulk solution, although only minimally influencing the capacity of the metal to catalyze a Fenton reaction, prevents the metal from doing so at the metal-binding site, thus inhibiting highly localized protein oxidation (B. Halliwell et al., *Arch. Biochem. Biophys.*, **246**:501-514 (1986); E. Stadtman, *Ann. Rev. Biochem.*, **62**:797-821 (1993)). We therefore evaluated the effects of metal chelators on the fragmentation reactions. Fe-induced cleavages were markedly inhibited by 1mM desferroxamine (Figure 3A, lanes 2 & 4), 1mM D-penicillamine (Figure 3A, lanes 2 & 8), or 1mM EDTA. Cu-induced fragmentation was entirely abolished by 200μM bathocuproine disulfonate (a Cu(I)-specific chelator; Figure 3A, lanes 3 & 7), 1mM EDTA, and was diminished by 1mM desferroxamine or 1mM D-penicillamine (Figure 3A, lanes 3, 5 & 9). These data strongly indicate that metal binding to the autoantigen itself is required for the fragmentation reactions to occur.

Since fragmentation requires exogenous metals capable of supporting a Fenton reaction, we addressed whether zinc, a metal that potentially binds to the same site, but is unable to support Fenton chemistry, might influence the ability of Fe or Cu to induce oxidative fragmentation. Increasing concentrations of zinc were added to cell lysates prior to the addition Fe/ascorbate, and the effects on autoantigen fragmentation were assessed (Figure 3B). Cleavage of topoisomerase I and RNA polymerase II large subunit was inhibited by zinc in a dose-dependent manner, with fragmentation entirely abolished by 300μM zinc (IC_{50} of 30-50μM) (Figure 3B). A similar IC_{50} for inhibition by zinc was observed for Cu/H_2O_2-induced fragmentation of these

autoantigens; in contrast, the specific aggregation of RNA polymerase II large subunit induced by Cu/H_2O_2 was not prevented.

Interestingly, a striking structural feature common to these fragmented autoantigens is extended charged tracts, containing regions rich in acidic residues, or regions of mixed charge (V. Brendel et al., *Proc. Natl. Acad. Sci. USA*, 88:1536-1540 (1991)). For example, topoisomerase I contains a single highly charged (mixed) tract containing 72 residues, NOR90/UBF contains 2 highly negatively-charged tracts of 21 and 28 residues, U1-70kDa) a contains 2 highly charged (mixed) tracts containing 89 and 48 mixed residues, and the large subunit of RNA polymerase II contains 3 highly charged (mixed) tracts of 25, 17 and 12 residues. Since the carboxylate side-chains of acidic residues constitute excellent sites for metal chelation, we addressed whether chemical modification of the accessible carboxylates by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) might alter the metal-catalyzed fragmentation of these proteins. Lysates of HeLa cells were incubated with 5mM EDAC for 5 minutes prior to initiation of a metal-catalyzed oxidation reaction by addition of Fe and ascorbate. EDAC significantly decreased the specific cleavage of topoisomerase I (Figure 3C) and RNA polymerase II large subunit, indicating that negatively charged amino acid side-chains are involved in the fragmentation reaction. In contrast, treatment with either diethyl pyrocarbonate (1mM) or iodoacetamide (1mM) (which modify histidine and cysteine residues, respectively), failed to decrease metal-catalyzed autoantigen fragmentation. Taken together, the specific cleavage sites, inhibition by chelators and EDAC, and competition by zinc strongly suggest that the cleaved scleroderma autoantigens possess metal-binding sites, which focus metal-catalyzed oxidation and resulting fragmentation at specific places. Since neither γ-irradiation (which generates OH by radiolysis of H_2O), nor addition of H_2O_2 or ascorbate alone to control lysates results in autoantigen fragmentation (Figure 1), it is likely that the physiologic state of these sites is either unoccupied, or occupied by a metal incapable of supporting Fenton chemistry.

Example 5- Specific autoantigen fragmentation is induced in intact cells chronically exposed to supraphysiologic concentrations of copper.

As with the absolute dependence of autoantigen fragmentation on exogenous Fe or Cu in cell lysates, similar autoantigen fragmentation can be induced in intact

cells chronically exposed to supraphysiologic concentrations of free Cu. Human foreskin keratinocytes were cultured for 18 hours *in vitro* in defined, serum- and albumin-free growth medium supplemented with 20μM $CuSO_4$. Oxidation reactions were initiated by adding 2mM H_2O_2 to some cultures for a further 30 minutes. This choice of cell type, metal ion and concentration, and H_2O_2 dose were based on preliminary studies which demonstrated that (a) loading cells with Fe was not feasible; (b) serum-free medium greatly facilitates the reproducible uptake of Cu; and (c) 20μM Cu (with H_2O_2) induces very little fragmentation in the *in vitro* lysate system. Only intact topoisomerase I was detected in control keratinocytes (Figure 4, lane 1), and H_2O_2 treatment alone did not result in any autoantigen fragmentation (Figure 4, lane 2). Incubation with 20μM Cu overnight did not affect cell morphology or viability, and did not produce any fragmentation of topoisomerase I in the absence of added H_2O_2 (Figure 4, lane 3). In contrast, addition of H_2O_2 after overnight Cu loading resulted in the marked fragmentation of topoisomerase I into the predominant Cu-characteristic band of 95kDa (Figure 4, lane 4). Incubation of cells in medium containing 20μM Cu for 2 hours generated no topoisomerase I fragments in the presence or absence of H_2O_2 (Figure 4, lanes 5 & 6), clearly demonstrating that overnight preincubation of cells with Cu sensitizes cells for H_2O_2-induced fragmentation of topoisomerase I. Washing away the Cu-containing medium prior to adding H_2O_2 had little effect on the extent or characteristics of fragmentation, indicating that the Cu-effect was cell-associated. Since addition of 20μM Cu and H_2O_2 fails to induce autoantigen fragmentation in cell lysates, the fragmentation observed in the intact cells pre-incubated with this Cu concentration strongly implies that higher localized concentrations of Cu are generated in these cells, which facilitate binding of Cu to the relevant autoantigens.

The most likely subcellular site in which these increased metal concentrations occur is the nucleolus, since the scleroderma autoantigens are enriched in nucleoli (reviewed in (E. Tan, *Cell*, **67**:841-842 (1991)), and several histochemical studies have demonstrated that the nucleolus has the unusual capacity to concentrate metal ions, including silver, zinc, cobalt and lead (C. Tandler, *J. Histochem. Cytochem.*, **4**:331-340 (1956); E. Pihl, *J. Microscopie*, **7**:509-520 (1968); G. Studzinski, *J. Histochem. Cytochem.*, **13**:365-375 (1965); C. Tandler et al., *J. Cell Biol.*, **41**:91-108

(1969)). Morphologic studies were performed to evaluate whether the patterns of staining of scleroderma autoantigens and metals in the nucleolus were similar (Figure 5A and 5B). Exclusive staining of punctate intranucleolar structures (nucleolini) was observed when HeLa cells were fixed with lead acetate and stained with ammonium sulfide (Figure 5A), or when ethanol-fixed HeLa cells were incubated with a solution containing zinc ions, prior to staining with dithizone (G. Studzinski, *J. Histochem. Cytochem.*, **13**:365-375 (1965)). Identical punctate intranucleolar structures were observed when confocal immunofluoresence microscopy was performed with antibodies to topoisomerase I (Figure 5B) and other scleroderma autoantigens.

Interestingly, effective autoantigen fragmentation results after prolonged incubation of intact cells with concentrations of extracellular Cu that are unable to induce protein fragmentation in cell lysates. The preincubation required suggests several possibilities, including the development of localized increases in the Cu concentration within the cell over time, and/or the slow exchange of Cu for a metal already bound to the autoantigen. The observation that scleroderma autoantigens are enriched in nucleoli (reviewed in (E. Tan, *Cell*, **67**:841-842 (1991)), Figure 5B), where they co-localize with sites of high-affinity metal binding (G. Studzinski, *J. Histochem. Cytochem.*, **13**:365-375 (1965); G. Studzinski et al., *Stain. Technol.*, **39**:397-401 (1964)), and Figure 5A), is therefore of great interest. It suggests that these antigens are unified both by their capacity to bind metals and their location at sites containing potentially increased local concentrations of redox active metals. A therapeutic application of the invention therefore preferably involves delivering fragmentation inhibitors to the nucleoli.

All documents are mentioned herein are incorporated herein by reference in their entirety.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modification and improvements within the spirit and scope of the invention as set forth in the following claims.

WHAT IS CLAIMED IS:

1. A method of treating a mammal suffering from or susceptible to an autoimmune disease, the method comprising administering to the mammal a therapeutically effective amount of a proteolytic inhibitor compound.

2. The method of claim 1, wherein the autoimmune disease is selected from the group consisting of scleroderma, multiple sclerosis, myasthenia gravis, polymyositis, graft-versus-host disease, graft rejection, raves disease, Addison's disease, autoimmune uveoretinitis, pemphigus vulgaris, primary biliary cirrhosis, rheumatoid arthritis, psoriasis, atopic dermatitis, osteoarthritis, Hashimoto's thyroiditis, primary myxoedema, thyrotoxicosis, pernicious anemia, autoimmune atrophic gastritis, insulin-dependent diabetes mellitus, Goodpasture's syndrome, pemphigoid, sympathetic opthalmia, phacogenic uveitis, autoimmune haemolytic anemia, idiopathic thrombocytopenic purpura, idiopathic leucopenia, active chronic hepatitis, cryptogenic cirrhosis, ulceratiye colitis, Sjogren's syndrome, dermatomyositis, mixed connective tissue disease, discoid lupus erythematosus, and systemic lupus erythematosus.

3. The method of claim 2, wherein the proteolytic inhibitor compound is administered to the mammal orally, intramuscularly, intraperitoneally or in an aerosol format.

4. The method of claim 3, wherein the proteolytic inhibitor compound is zinc or a metal chelator.

5. The method of claim 4, wherein the metal chelator is selected from the group consisting of desterroxamine, D-penicillamine, ethylenediamine tetraacetic acid (EDTA), and bathocuproine disulfonate.

6. The method of any one of claims 1-5, wherein the proteolytic inhibitor compound inhibits production of at least one autoantigenic and immunogenic protein fragment by at least 10% in a standard protein fragment detection assay, the protein fragment being produced from an autoantigen by metal-catalyzed oxidative proteolysis.

7. The method of any one of claims 1-5, wherein the proteolytic inhibitor compound has an IC_{50} of between about 0.1μM and 100μM.

8. A method of treating a mammal suffering from or susceptible to an autoimmune disease, the method comprising administering to the mammal a therapeutically effective amount of an autogenic and immunogenic protein fragment produced by metal-catalyzed oxidative proteolysis of an autoantigen.

9. The method of claim 8, wherein the autoantigen is a nucleic acid binding protein.

10. The method of claim 9, wherein the autoantigen is selected from the group consisting of a topoisomerase, RNA polymerase, upstream binding factor (UBF/NOR90), and the U1 small nuclear ribonucleoprotein.

11. The method of claim 8, wherein the autoimmune disease is selected from the group consisting of scleroderma, multiple sclerosis, myasthenia gravis, polymyositis, graft-versus-host disease, graft rejection, raves disease, Addison's disease, autoimmune uveoretinitis, pemphigus vulgaris, primary biliary cirrhosis, rheumatoid arthritis, psoriasis, atopic dermatitis, osteoarthritis, Hashimoto's thyroiditis, primary myxoedema, thyrotoxicosis, pernicious anemia, autoimmune atrophic gastritis, insulin-dependent diabetes mellitus, Goodpasture's syndrome, pemphigoid, sympathetic opthalmia, phacogenic uveitis, autoimmune haemolytic anemia, idiopathic thrombocytopenic purpura, idiopathic leucopenia, active chronic hepatitis, cryptogenic cirrhosis, ulceratiye colitis, Sjogren's syndrome, dermatomyositis, mixed connective tissue disease, discoid lupus erythematosus, and systemic lupus erythematosus.

12. The method of claim 8, wherein the proteolytic inhibitor compound is administered to the mammal orally, intramuscularly, intraperitoneally or in an aerosol format.

13. The method of claim 8, wherein the autoantigenic and immunogenic protein fragment is administered in combination with another therapeutically effective compound.

14. The method of claim 13, wherein the other therapeutically effective compound is a proteolytic inhibitor compound.

15. The method of claim 14, wherein the proteolytic inhibitor compound is zinc or a metal chelator.

16. The method of claim 15, wherein the metal chelator is selected from the group consisting of desterroxamine, D-penicillamine, ethylenediamine tetraacetic acid (EDTA), and bathocuproine disulfonate.

17. The method of claims 8-13, wherein the autoantigenic and immunogenic protein fragment tolerizes an immune response directed against the protein fragment by the mammal.

18. An autoantigenic and immunogenic fragment of an autoantigen produced by metal-catalyzed oxidative proteolysis, the fragment having a molecular weight of between about 10kDa to 500kDa.

19. A method of making an autoantigenic and immunogenic fragment of an autoantigen having a molecular weight of between about 10kDa to 500kDa, the method comprising exposing the autoantigen to metal-catalyzed oxidative proteolysis under conditions sufficient to produce the protein fragment.

20. The method of claim 18, wherein the autoantigen is a nucleic acid binding protein.

21. The method of claim 19, wherein the nucleic acid binding protein is selected from the group consisting of topoisomerase I, large subunit of RNA polymerase II, upstream binding factor (UBF/NOR90), and U1 small nuclear ribonucleoprotein (U1-70kDa).

22. An antibody capable of specifically binding the protein fragment of claim 20.

23. A method of detecting an autoimmune disease in a mammal comprising obtaining a biological sample from a mammal, contacting the sample with the antibody of claim 22 under conditions suitable for forming a specific binding complex between the antibody and one or more autoantigenic and immunogenic protein fragments; and detecting formation of the specific binding complex as indicative of the autoimmune disease in the mammal.

24. A method of detecting an autoimmune disease in a mammal comprising obtaining a biological sample from a mammal comprising or suspected of comprising an autoantibody, contacting the sample to one or more autoantigenic and immunogenic protein fragments produced by metal-catalyzed oxidative proteolysis, the contacting being under conditions suitable for forming a specific binding complex

between the an the autoantibody and one or more of the protein fragments; and detecting formation of the specific binding complex as indicative of the autoimmune disease in the mammal.

25. A method for determining therapeutic capacity of a candidate inhibitor compound for treating an autoimmune disease, the method comprising:

1) culturing a population of cells in medium comprising one or more Fenton metals in an amount sufficient to support metal-catalyzed oxidative proteolysis;

2) adding a candidate inhibitor compound to the medium;

3) measuring production of at least one autoantigenic and immunogenic protein fragment by the cells; and

4) determining the effect of the candidate inhibitor compound on the production of the protein fragments.

26. A method for determining therapeutic capacity of a candidate inhibitor compound for treating an autoimmune disease, the method comprising:

1) preparing a lysate from a population of cultured cells;

2) adding a candidate inhibitor compound to the lysate;

3) adding at least one Fenton metal to the lysate in an amount sufficient to support metal-catalyzed oxidative proteolysis;

4) measuring production of at least one autoantigenic and immunogenic protein fragment in the lysate; and

5) determining the effect of the candidate inhibitor compound on the production of the protein fragments in the lysate.

27. A method of treating an autoimmune disease comprising administering an effective amount of a substance that inhibits the site-specific chemical fragmentation of autoantigens into peptides in the presence of metals such as iron or copper and reactive oxygen species; or a administering antigenic protein fragments to tolerize the patient's immune system.

28. The method of claim 27 in which the autoimmune disease is scleroderma, the autoantigens are selected from the group consisting of topoisomerase I, the large subunit of RNA polymerase II, upstream binding factor (UBF/NOR90), and the 70kDa protein component of the U1 small nuclear ribonucleoprotein, and the

substance is selected from the group consisting of desferroxamine, D-penicillamine, 1mM EDTA, bathocuproine disulfonate, and other chelators; zinc and other metals and agents that compete with the iron or copper; chemicals that modify the accessible carboxylates such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; and Fenton oxidation protease inhibitors.

29. A composition useful in reducing injury from an autoimmune disease comprising an effective amount of a substance that inhibits the site-specific non-enzymatic fragmentation of autoantigens into peptides in the presence of metals such as iron or copper and reactive oxygen species.

30. Scleroderma autoantigen fragments selected from the group consisting of: topoisomerase I fragments migrating at 95kDa, and species migrating between 65kDa and 90kDa; fragments of the large subunit of RNA polymerase II migrating at 190, 160 and 140kDa, and fragments migrating at 200, 180, 170 and 130kDa; fragments of the upstream binding factor (UBF/NOR90) migrating 70kDa and 90kDa; and fragments of 70kDa protein component of theU1 small nuclear ribonucleoprotein (U1-70kDa) migrating between 33 and 38kDa.

31. A method of making scleroderma autoantigens comprising obtaining a protein selected from the group consisting of topoisomerase I, the large subunit of RNA polymerase II, upstream binding factor (UBF/N0R90), and the 70kDa protein component of the U1 small nuclear ribonucleoprotein, and fragmenting the protein in the presence of iron or copper and reactive oxygen species.

32. An assay for scleroderma comprising an antibody to a fragment of a scleroderma autoantigen according to claim 30.

33. A method for diagnosing scleroderma comprising obtaining an antibody to a fragment of a scleroderma autoantigen according to claim 30, reacting it with a sample from a patient, and detecting whether the scleroderma autoantigen binds to the antibody.

34. A method for diagnosing an autoimmune disease comprising obtaining an antibody to an autoantigenic protein fragment of an autoantigen of that disease that is subject to cleavage by a metal-catalyzed reaction, reacting it with a sample from a patient, and detecting whether the autoantigen binds to the antibody.

35. A screening assay for a drug with activity against an autoimmune disease, comprising:

establishing a control system comprising combining a self-tolerated autoantigen of the autoimmune disease with iron or copper or other metal catalyst and reactive oxygen species, fragmenting the autoantigen, thereby producing a decrypted autoantigenic protein fragment, and detecting the decrypted fragment with an antibody to the fragment;

establishing a test system comprising adding a drug to the control system and determining whether the drug reduces the producing of a decrypted autoantigenic fragment; and

comparing the results of the control system and the test system to determine whether a particular drug is effective against the autoimmune disease.

36. A method of making autoantigenic protein fragments comprising obtaining an autoantigenic protein susceptible to site-specific non-enzymatic cleavage in a metal catalyzed reaction, and fragmenting the protein in the presence of metal and reactive oxygen species.

37. A composition comprising an autoantigenic protein fragment produced from an autoantigenic protein by site-specific non-enzymatic cleavage in a metal catalyzed reaction.

38. An assay for an autoimmune disease comprising an antigenic protein fragment according to claim 37.

39. An assay for an auto immune disease comprising an antibody to an antigenic protein fragment according to claim 37.

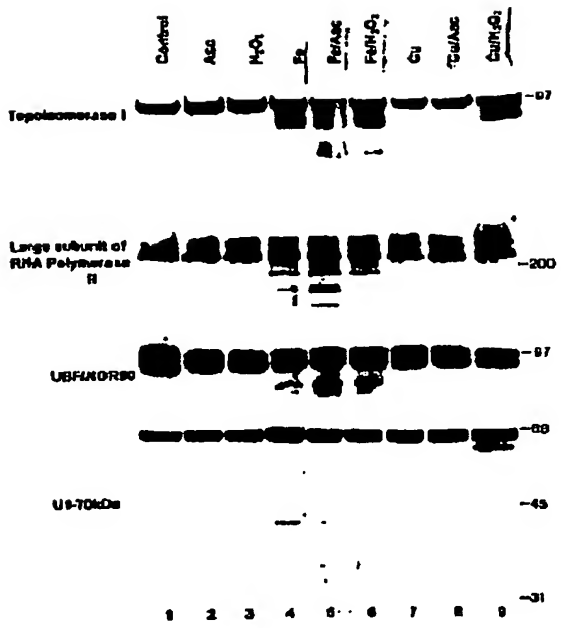
Control
Asc
H2O2
Fe
Fe/Asc
Fe/H2O2
Cu
Cu/Asc
Cu/H2O2
Topoisomerase I
Large subunit of RNA Polymerase II
UBF/NORS90
U3-70kDa
97
200
97
69
45
31
1 2 3 4 5 6 7 8 9

FIGURE 1

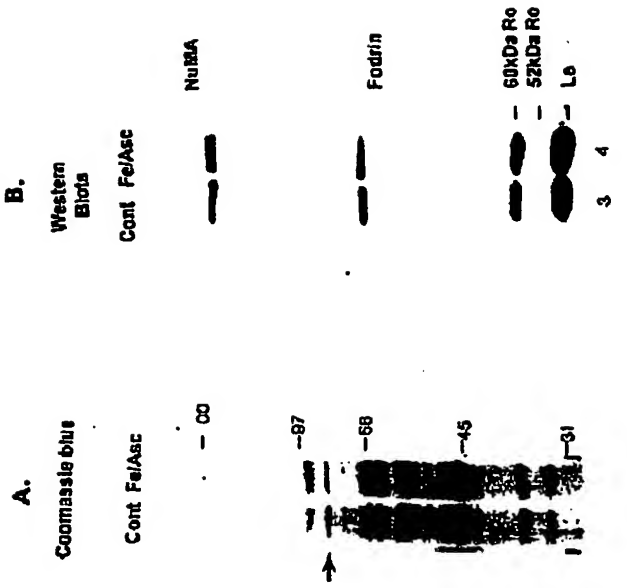

FIGURE 2 B

FIGURE 2 A

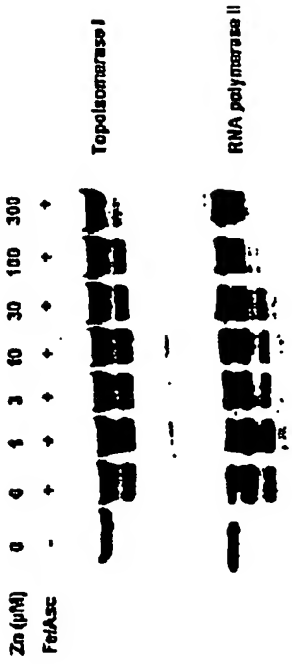

FIGURE 3 B

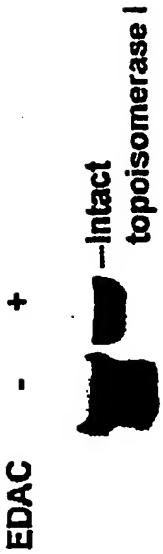

FIGURE 3 C

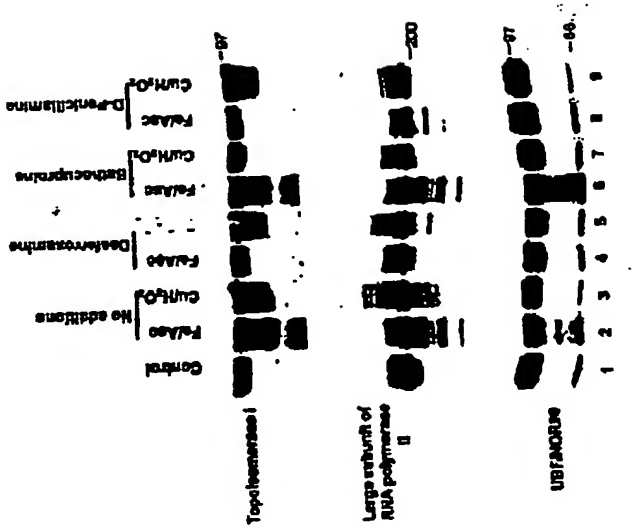

FIGURE 3 A

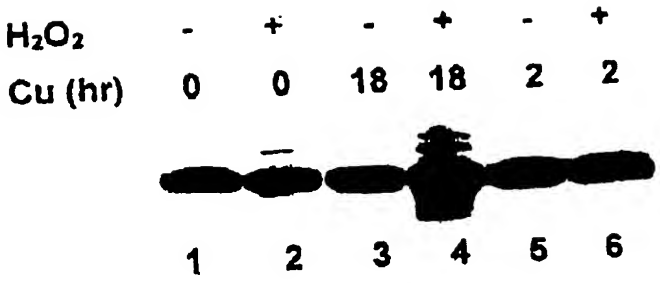

FIGURE 4

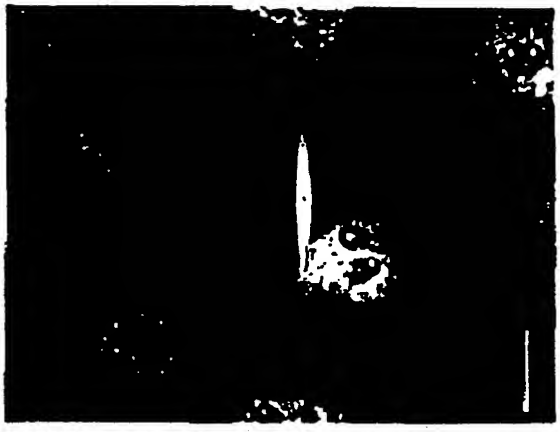

FIGURE 5 B

FIGURE 5 A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24100

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 198.1; 435/7.1; 436/506, 508, 547, 811; 514/1, 885, 886; 530/309, 345, 402, 388.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,583,153 A (BRAHN) 10 December 1996, see entire document.	1-7, 12-16, 27-29
Y	US 5,070,192 A (EARNSHAW et al.) 03 December 1991, see entire document.	8-11, 17-24, 30, 32-34, 37-39
Y	US 5,571,500 A (HAFLER et al.) 05 November 1996, see entire document	8-11, 17-24, 30, 32-34, 37-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents.
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 MARCH 1998	13 APR 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24100

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 31/00, 39/00, 38/00, 38/17; G01N 33/53, 33/564, 37/00; C07K 14/00, 14/435, 16/18, 1/107

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/184.1, 198.1; 435/7.1; 436/506, 508, 547, 811; 514/1, 885, 886; 530/300, 345, 402, 388.2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE, DERWENT WPI, CHEM AB, APS, search terms: author names, autoimmune, proteolytic inhibitor, zinc or metal chelator, compounds of claim 5, autoantigen, metal catalyzed, antibody, fenton metal, scleroderma